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Microarray analysis of acaricide-inducible gene expression in the southern cattle tick, *Rhipicephalus (Boophilus) microplus*

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Abstract

Acaricide-inducible differential gene expression was studied in larvae of *Rhipicephalus (Boophilus) microplus* using a microarray-based approach. The acaricides used were: coumaphos, permethrin, ivermectin, and amitraz. The microarrays contained over 13 000 probes, having been derived from a previously described *R. microplus* gene index (BmiGI Version 2; Wang *et al.*, 2007). Relative quantitative reverse transcriptase-PCR, real time PCR, and serial analysis of gene expression data was used to verify microarray data. Among the differentially expressed genes with informative annotation were legumain, glutathione S-transferase, and a putative salivary gland-associated protein.

Keywords: *Rhipicephalus (Boophilus) microplus*, acaricide resistance, organophosphates, microarrays, detoxification enzymes.

Introduction

One of the biggest arthropod threats to livestock is the ectoparasite *Rhipicephalus (Boophilus) microplus*, also referred to as the Tropical/ Southern Cattle Tick. *R. microplus* serves as a vector for the pathogenic protozoan which causes bovine babesiosis, also known as cattle fever, a severe and often fatal disease to cattle. Before 1906, direct and indirect economic losses to the U.S. cattle industry attributable to this disease were estimated to be $130.5 million which, if adjusted for inflation, would be approximately $3 billion in today's dollars (APHIS, 2002). In 1906, the United States Department of Agriculture (USDA) was responsible for the inception of the Cattle Fever Tick Eradication Program (CFTEP) (Graham & Hourrigan, 1977). The CFTEP has been extremely successful in averting the reintroduction of *R. microplus* into the U.S. from Mexico where *R. microplus* is endemic and still causes severe economic damage to Mexican ranchers. The CFTEP relies heavily on physical inspection of livestock presented at the U.S.-Mexico border for importation and the application of chemical acaricides, administered in organophosphate (OP)-charged dipping vats to over a million head of imported cattle per year (USDA-ERS Data Sets, 2006).

Populations that are resistant to OP and/or pyrethroid acaricides have been observed in Mexico for a number of years (Fragoso *et al.*, 1995), and resistance to the formamidine pesticide amitraz has been observed in Mexico within the last few years (Rodríguez-Vivas *et al.*, 2006). Recently, acaricide resistant population outbreaks of *R. microplus* have been found in the United States (Miller *et al.*, 2005, 2007). There is a pressing need to monitor the magnitude and mechanisms of pesticide resistance in Mexican tick populations and outbreak populations within the U.S. to assist the CFTEP in maintaining the *Boophilus*-free status of the U.S. Molecular-based assays exist to detect target site modifications which lead to pyrethroid and OP resistance. A PCR assay is available which detects the presence of a pyrethroid resistance-causing gene mutation in the tick sodium channel, the target of pyrethroids (Guerrero *et al.*, 2001). OP-insensitive acetylcholinesterase, the target of OPs, is detectable through a comparative inhibition kinetics biochemical assay (Pruett, 2002). However, acaricide resistance can also occur due to metabolic sequestration or detoxification of the acaricide and these

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resistance mechanisms have been less studied at the molecular level than target site mechanisms. The existence of the metabolic detoxification resistance mechanism has been documented in the *R. microplus* Coatzacoalcos strain from Mexico through the use of larval packet test bioassays in the presence and absence of the metabolic esterase-inactivating synergist, triphenyl phosphate (Miller *et al*., 1999) and native esterase activity gel assays (Jamroz *et al*., 2000). Miller *et al*. (1999) used synergist bioassay studies to document the contributions that mixed function oxidase-, metabolic esterase-, and glutathione transferase-based detoxification mechanisms make to the overall resistance phenotype in various strains of *R. microplus*. Metabolic detoxification is also a likely component of the resistance mechanism of the Uberlandia strain from Brazil (Baffi *et al*., 2007).

The availability of an expressed sequence tag (EST) database for *R. microplus*, BmiGI (Guerrero *et al*., 2005; Wang *et al*., 2007; http://compbio.dfci.harvard.edu/tgi), presents the opportunity to study gene expression in *R. microplus* at a more global level than previously possible. BmiGI Version 2 contains 13 643 unique sequences, a significant fraction of this tick's complement of gene coding regions, including 9403 tentative consensus (TC) sequences and 4240 unassembled singletons, produced from a total of 42 512 ESTs. The source material for these ESTs consisted of polyA RNA from various tissues, life stages, and strains of *R. microplus*, including larvae exposed to heat, cold, host odor, and acaricides. Additionally, RNA isolated from larvae of acaricide resistant strains was part of the source material. With our interest in supporting the CFTEP, we desired to further our insight into acaricide resistance mechanisms by examining changes in gene expression which occur upon acaricide exposure. A microarray-based approach using array probes synthesized from the sequences in BmiGI Version 2 seemed most appropriate. Vontas *et al*. (2005) used a microarray-based approach to identify constitutive and insecticide-inducible differential gene expression in susceptible and insecticide resistant mosquitoes. Both up- and down-regulated constitutive and permethrin-inducible gene expression was detected in genes with various functions in the mosquito. Thus, we expected to see both up- and down-regulation of tick gene expression in response to acaricide exposures. We exposed an amitraz resistant Mexican strain of *R. microplus* to low doses of coumaphos, permethrin, amitraz and ivermectin and compared gene expression, as quantified by the BmiGI Version 2 microarrays, between the acaricide exposed samples and unexposed control. As a method of verifying the microarray data, we used relative quantitative reverse transcriptase-PCR of selected differentially regulated transcripts. We also compared the microarray results from the coumaphos exposed larvae to results from libraries of Serial Analysis of Gene Expression (SAGE; Velculescu *et al*., 1995) tags from a previous study of coumaphos-exposed larvae (Guerrero *et al*., 2007).

Table 1. Acaricide treatments and associated mortality

Acaricide	Resistance*	Dose†	Larval Mortality
Coumaphos	2-fold	0.07%	7.5%
Permethrin	91-fold‡	0.07%	20.4%
Amitraz	> 600-fold	0.07%	14.6%
Ivermectin	(no data)	0.20%	13.4%

*% Resistance relative to susceptible Munoz strain determined by larval packet bioassays (FAO, 1971; R. Miller, personal communication).
†% active ingredient.
‡Data for Permethrin not available, stated resistance is for Flumethrin.

Results and discussion

Differential expression data analysis

Significance Analysis of Microarrays and clustering. To study acaricide-inducible gene expression in *R. microplus*, larvae were treated with low doses of acaricide in filter paper packet tests and mortality level determined by counting dead larvae after 24 h exposure (Table 1). Doses were selected based on 50% lethal dose (LD_{50}) bioassay determinations on the previous generation of the San Alfonso strain (data not shown), attempting to induce low mortality rates of 10−20% during each exposure. All larvae were suctioned into sample tubes and RNA was isolated and prepared for microarray hybridization. Microarray data analysis was performed using Significance Analysis of Microarrays (SAM). SAM reduces the false positive rates in analysis of large datasets such as those found in microarray experiments. As determined by the SAM algorithm, the total number of statistically significant up-regulated transcripts for coumaphos, permethrin, ivermectin, and amitraz were 27, 16, 28, and 32, respectively (see Supporting Information). Results for the down-regulated coumaphos, permethrin, ivermectin, and amitraz treatment transcripts were 49, 16, 52, and 51, respectively (see Supporting Information). This tick strain is amitraz resistant and the highest total number of significantly differentially expressed genes (83) was seen in the amitraz exposed sample. This strain was collected from an area where exposure and resistance to ivermectin was very unlikely. However, the ivermectin treated sample showed the second highest number of differentially expressed genes (80). Thus a correlation between the number of differentially expressed genes upon acaricide exposure and phenotypic acaricide resistance does not seem likely.

In looking for overall patterns of inducible gene expression, the lack of sequence similarity between *R. microplus* genes and genes from organisms with well-annotated genomes was a major handicap. SAM detected 103 significantly up-regulated transcripts and 168 down-regulated

Table 2. Seven most significantly up regulated members of BmiGI Version 2 following acaricide exposure

			BlastX Annotation			
BmiGI Ver2 ID*	d†	FC‡	ID	Species	Acc. No.	E-value
Control vs. coumaphos						
TC9408	19.1	16.5	No significant similarity found	–	–	–
TC6631	13.6	8.9	Plancitoxin-1 precursor	*Acanthaster planci*	BAD13432.1	4e–56
TC8762	12.9	8.3	No significant similarity found	–	–	–
TC11562	17.0	7.4	Hypothetical protein	*Burkholderia phytofirmans PsJN*	ZP_01509121.1	2e–04
TC9771	12.2	5.9	Salivary gland-associated prot. 64P	*Rhipicephalus appendiculatus*	AAM09648.1	5e–04
TC14558	13.0	5.7	Cell adhesion prot. sym32	*Anthopleura elegantissima*	AAF65308.1	1e–21
TC8947	15.9	4.8	No significant similarity found	–	–	–
Control vs. permethrin						
TC9408	20.0	11.8	No significant similarity found	–	–	–
BEAC749TF	15.1	7.4	No significant similarity found	–	–	–
TC9771	13.6	6.7	Salivary gland-associated prot. 64P	*Rhipicephalus appendiculatus*	AAM09648.1	5e–04
TC11562	18.1	5.9	Hypothetical protein	*Burkholderia phytofirmans PsJN*	ZP_01509121.1	2e–04
TC6022	11.7	5.7	Chorion b-ZIP transcription factor	*Bombyx mori*	NP_001037099.1	1e–10
TC14558	12.6	4.5	Cell adhesion prot. sym32	*Anthopleura elegantissima*	AAF65308.1	1e–21
TC12018	13.3	4.1	Tick legumain	*Haemaphysalis longicornis*	BAF51711.1	8e–172
Control vs. ivermectin						
TC9771	19.4	10.2	Salivary gland-associated prot. 64P	*Rhipicephalus appendiculatus*	AAM09648.1	5e–04
TC9408	18.4	8.6	No significant similarity found	–	–	–
TC8947	37.2	8.4	No significant similarity found	–	–	–
TC6022	16.7	7.7	Chorion b-ZIP transcription factor	*Bombyx mori*	NP_001037099.1	1e–10
TC14692	16.0	5.3	No significant similarity found	–	–	–
TC8948	22.7	4.9	Hypothetical protein	*Danio rerio*	XP_0001339406.1	4e–04
TC5786	23.2	4.6	No significant similarity found	–	–	–
Control vs. amitraz						
TC9408	15.1	14.0	No significant similarity found	–	–	–
TC7207	13.9	10.9	Microplusin preprotein-like	*Ixodes scapularis*	AAY66495.1	1e–05
TC9771	12.9	9.7	Salivary gland-associated prot. 64P	*Rhipicephalus appendiculatus*	AAM09648.1	5e–04
TC14511	11.6	8.8	No significant similarity found	–	–	–
BEAC749TF	12.7	8.8	No significant similarity found	–	–	–
TC6022	11.7	7.3	Chorion b-ZIP transcription factor	*Bombyx mori*	NP_001037099.1	1e–10
TC12018	11.9	5.2	Tick legumain	*Haemaphysalis longicornis*	BAF51711.1	8e–172

*BmiGI Ver2 ID represents the identification number from BmiGI Version 2; †d is the d statistic as performed by SAM; ‡FC is the fold change ratio.

transcripts. However, only 18 and 13 of these transcripts from the up- and down-regulated sets, respectively, had low enough BlastX E-values to assign putative gene product function. Tables 2 and 3 report the 7 most up- and down-regulated BmiGI transcripts, respectively, following exposure to each of the 4 pesticides used in our study. Twelve of the up-regulated transcripts in Table 2 and all 28 of the down-regulated transcripts in Table 3 have no significant BlastX hits (E-value < 0.001). In an attempt to gain information on the role of these transcripts in the tick, we used the prot4est translations of these 40 BmiGI transcripts (Wang *et al*., 2007) to perform a domain search using the National Center for Biotechnology Information's Conserved Domain Database and reverse position-specific-Blast algorithm with E-value < 0.01 (Marchler-Bauer *et al*., 2007; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). However, none of the searches found conserved domains in these 40 unannotated transcripts using the CDD database containing 24 291 position-specific score matrices (data not shown).

One common theme for all four acaricide treatments was the up-regulation of at least one well-annotated gene whose function likely involves bloodmeal digestion (see Supporting Information). These ranged from the digestive peptidase legumain (TC12017 and TC12018) to the thrombin inhibitor represented by TC9992, discussed in more detail below. The down-regulated transcripts were predominately BmiGI singletons, as 146 of the 168 differentially down-regulated transcripts corresponded to BmiGI sequences which did not assemble into TCs. As such, these unassembled sequences likely represent low abundance tick transcripts. With so few down-regulated transcripts possessing useful annotation, common themes in acaricide response in this dataset were even more difficult to discern than the up-regulated transcript dataset.

Hierarchical clustering (Eisen *et al*., 1998) by sample was performed on the microarray datasets as a data quality check to verify the samples would cluster within their respective treatment groups. Clustering of sample replicates within their respective treatment groups would be indicative that most variance is due to biological rather than technical factors. If technical variation were higher than biological variation, clustering within their respective treatment

Table 3. Seven most significantly down regulated members of BmiGI Version 2 following acaricide exposure

BmiGI Ver2 ID*	d†	FC‡	BlastX Annotation
Control vs. coumaphos			
BEAF613TF	−15.0	−7.4	No significant similarity found
BEAA077TF	−20.4	−7.0	No significant similarity found
BEADG74TF	−14.9	−6.9	No significant similarity found
BEAEO96TF	−14.6	−6.9	No significant similarity found
BEAAW55TF	−15.1	−6.6	No significant similarity found
BEAE046TF	−15.6	−6.2	No significant similarity found
BEADV09TF	−16.2	−5.2	No significant similarity found
Control vs. permethrin			
BEADD60TF	−11.8	−6.6	No significant similarity found
BEAFZ74TR	−10.8	−5.8	No significant similarity found
BEAEV13TF	−13.6	−5.5	No significant similarity found
BEAE046TF	−11.0	−5.2	No significant similarity found
BEAEP23TF	−13.8	−5.1	No significant similarity found
BEAFC55TR	−13.0	−4.3	No significant similarity found
BEACP60TF	−11.0	−4.1	No significant similarity found
Control vs. ivermectin			
BEAF613TF	−15.7	−8.4	No significant similarity found
BEAA077TF	−28.4	−7.6	No significant similarity found
BEAE046TF	−16.2	−7.0	No significant similarity found
BEAEO96TF	−20.1	−6.4	No significant similarity found
BEADV09TF	−22.2	−5.5	No significant similarity found
BEAAK71TF	−19.1	−4.4	No significant similarity found
TC10388	−16.2	−4.1	No significant similarity found
Control vs. amitraz			
BEAAR47TF	−13.5	−12.0	No significant similarity found
BEAA077TF	−16.2	−8.8	No significant similarity found
BEAAW55TF	−13.6	−7.9	No significant similarity found
BEADG74TF	−12.3	−7.8	No significant similarity found
BEAE983TF	−13.1	−7.5	No significant similarity found
BEAE046TF	−13.9	−7.2	No significant similarity found
BEADV09TF	−12.2	−6.0	No significant similarity found

*BmiGI Ver2 ID represents the identification number from BmiGI Version 2; †d is the d statistic as performed by SAM; ‡FC is the fold change ratio.

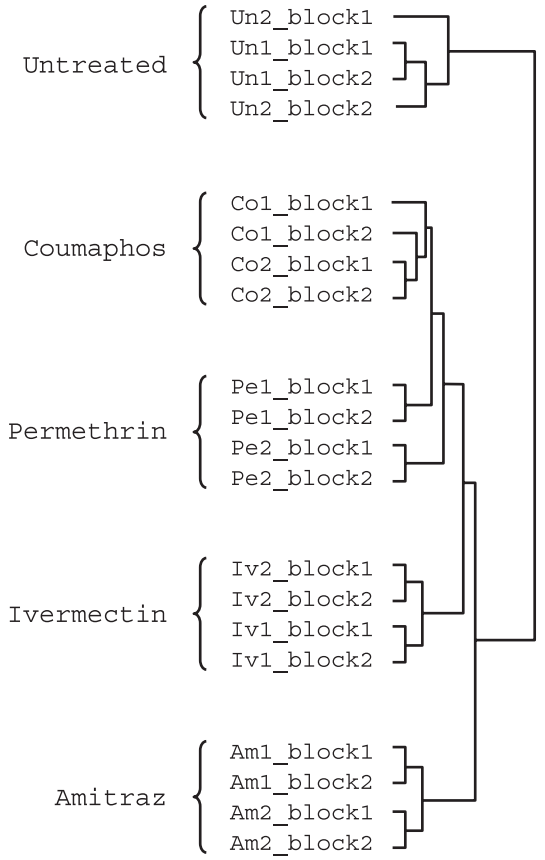

Figure 1. Acaricide-exposed mRNA microarray dataset hierarchically clustered by sample. A variance filter which removed ESTs with low variation of expression over the samples was used and only the top 10% of the most differentially expressed ESTs were used for clustering. Euclidean distance was the chosen distance metric and average linkage was the linkage method used. Un, Co, Pe, Iv, and Am signify untreated control, coumaphos, permethrin, ivermectin and amitraz treatments, respectively. The number following the treatment abbreviation indicates the two microarray replicate chips and BLOCK indicates the two within chip replicates.

groups would not occur. A variance filter was used so that only the top 10% most significantly differentially expressed genes were clustered, improving the sensitivity by removing genes which do not vary much in their expression over the range of treatments. All samples clustered within their respective treatment groups and the control was the most distant of all the sample groups (Fig. 1). The results can be depicted through the notation ((((coumaphos, permethrin) ivermectin) amitraz) control). The pattern of clustering showed coumaphos and permethrin are the most differentially expressed datasets relative to the control and the overall patterns of expression by the coumaphos and permethrin samples are more similar to each other than the other two acaricide treatment samples. The clustering also shows there was less within-chip than between-chip variation, due to the consistent clustering of spot (within-chip) replicates.

Significantly up-regulated transcripts. Table 2 lists the seven most up-regulated members of BmiGI Version 2 for each acaricide treatment as revealed by the microarrays. Several BmiGI members occur in more than one acaricide treatment list. TC9408 and TC9771 occur in all four lists while TC6022 occurs in three (permethrin, ivermectin, and amitraz) of the four top seven lists. Additionally, TC6022 ranks 10th among the most up-regulated BmiGI members in the coumaphos treatment (see Supporting Information). Thus, these three TCs all rank in the top ten list of most up-regulated BmiGI members for all four acaricide treatments, making it tempting to speculate they represent a group of xenobiotic-responsive genes. However, Blast analysis did not find any significant match to TC9408 and although TC9771 showed similarity to a salivary gland-associated protein, the E-value of the match is fairly high (2e−04). TC6022 shows sequence similarity to a chorion b-ZIP DNA-binding transcription factor from *Bombyx mori* (Sourmeli *et al*., 2005), but again the match is not strong (E-value = 1e–10). TC11562 (no useful annotation) and TC14558 (cell adhesion protein, E-value = 1e–21) are in the top seven most up-regulated lists for coumaphos and permethrin treatments. TC8947 (no useful annotation) is on

the top seven list for coumaphos and ivermectin treatments. Finally, TC12018 (legumain, E-value = 5e–172) and BEAC749TF (no useful annotation) are on the top seven lists for permethrin and amitraz treatments. Tick legumain, an asparaginyl endopeptidase, plays an important role in host blood-meal digestion and Alim *et al.* (2007) speculated legumain mediates other physiological processes associated with survival, development and reproduction in the tick. It is interesting that Vontas *et al.* (2005) found a microsomal aminopeptidase-like transcript (EST clone NAP1-P158-E-04) was down-regulated in *Anopheles gambiae* 6 and 10 h following exposure to permethrin. Conversely, two other ESTs from this group's study of *A. gambiae* gene expression which were annotated as associated with proteolysis (EST clone NAP1-P104-D-01) or protease activity (EST clone 4A3B-AAE-A-09) were up-regulated upon permethrin exposure.

Significantly down-regulated transcripts. Table 3 lists the seven most down-regulated members of BmiGI Version 2 for each acaricide treatment as revealed by the microarrays. Unfortunately, there were no significant similarities found between these down-regulated members of BmiGI and GenBank entries (E-value < 0.001). This underscores one difficulty frequently encountered during molecular studies of an arthropod whose genome contains a significant fraction of genes with limited sequence similarity to well-annotated genes (Wang *et al.*, 2007). In addition, all but one of the BmiGI members listed in Table 3 are singletons, indicating these were low abundance transcripts in the normalized cDNA library which served as the source material for BmiGI (Guerrero *et al.*, 2005). Coumaphos-, permethrin-, and amitraz-treated larvae were used as part of the sample to synthesize the BmiGI normalized library and acaricide-inducible genes were expected to be found in BmiGI. The low abundance of the singletons might also explain their lack of annotation. It is possible these are tick-specific genes having very limited sequence similarity to genes from non-tick species. Despite the lack of annotation, several BmiGI members appear to down-regulate their expression in response to exposure to acaricides from different classes perhaps serving as general xenobiotic responders. BEAE046TF is present in all four acaricide treatment lists in Table 3, while BEAA077TF and BEADV09TF are present in the coumaphos, ivermectin and amitraz treatment lists. BEAAW55TF and BEADG74TF were in the coumaphos and amitraz treatment lists while BEAF613TF and BEAE096TF were in the coumaphos and ivermectin lists. It should be noted that the normalized cDNA library which served as the source material for BmiGI did not include any ivermectin-exposed larvae. Consequently, our microarray which is designed from BmiGI might not be optimal for detecting transcripts which are exclusively induced or repressed by ivermectin exposure.

Microarray result verifications

Comparison to SAGE tag library data. We wanted to verify these microarray results and validate the arrays for future studies. Our first verification method made use of SAGE library databases from a previous study of coumaphos exposed *R. microplus* larvae (Guerrero *et al.*, 2007). SAGE produces a snapshot quantification of mRNA under the experimental conditions and tissues of interest and yields database libraries of short sequence tags of sufficient length to uniquely identify the transcript serving as the source of the tag. Tallies of tag frequency allow transcript quantification and comparisons of tag frequency between libraries can result in statistically-based data sets quantifying differential gene expression. The SAGE study used the susceptible reference Munoz strain and the San Roman strain, a Mexican strain possessing a fairly high level of acaricide resistance, including 8-fold resistance to coumaphos. Different molecular-based mechanisms of acaricide resistance might exist in San Roman compared to the San Alfonso strain. Nevertheless, we identified six members of BmiGI which had both SAGE library data and were differentially expressed in these microarray experiments using the statistical criteria noted in the Experimental Procedures section (Table 4). Unfortunately, four of the six did not have informative BlastX annotation and a fifth had a relatively high BlastX E-value (TC9771; 5e–04).

Table 4. Comparison of results from coumaphos up- and down-regulated microarray and SAGE coumaphos-treated data sets

			Munoz		San R		BlastX Annotation		
BmiGI ID*	d†	FC‡	U§	T¶	U§	T¶	ID	Acc. No.	E-value
TC6034	8.7	4.5	2	3	0	1	No significant similarity found	–	–
TC9004	9.5	4.4	1	5	0	2	Glutathione S-transferase	AAO92279.1	2e–78
TC9771	12.2	5.9	1	2	0	1	Salivary gland-ass. prot. 64P	AAM09648.1	5e–04
TC12769	9.7	4.9	0	0	0	1	No significant similarity found	–	–
TC14511	9.6	5.4	0	0	1	1	No significant similarity found	–	–
BEAFZ74TR	–10.2	–6.3	0	0	0	1	No significant similarity found	–	–

*BmiGI ID represents the identification number from BmiGI Version 2; †d is the d statistic as performed by SAM; ‡FC is the fold change ratio; §U represents SAGE tag counts from untreated larvae; ¶T represents SAGE tag counts from coumaphos-treated larvae.

Table 5. Relative quantitative RT-PCR of selected acaricide-induced BmiGI Version 2 members

		Microarray‡			RT-PCR quantification§				
BmiGI ID*	Primers†	Acaricide	d	FC	Un¶	Co	Pe	Am	Iv
TC6221	FW: GAACTGTGACAATCGCAGGAC	Am	9.5	4.6	1	0.91	0.65	1.46	1.20
	RV: CATTTACCGCGAGAGCTTGAAG								
TC6631	FW: GTACCCCAACATCTACGACAGC	Am	10.6	6.4	1	4.0	2.28	7.57	4.29
	RV: CATGCGAGCTTTATTTCCAGC	Iv	10.1	4.6					
		Co	13.3	8.9					
TC7207	FW: TAGGCTTCAGCTGTAGCCTC	Am	13.9	10.9	1	1	0.93	2.18	1.29
	RV: GAAACCACCTTGAATTTGAGTC	Co	8.4	4.1					
TC8948	FW: AGCGGAAGCTTCTGGGGAC	Iv	22.7	4.9	1	1.11	0.94	1.97	1.42
	RV: CCAGCTGAAGAAACTTCCACGTC								
TC9004	FW: CAACCCCGAGTTTGTCAAG	Co	9.5	4.4	1	1.67	1.25	2.75	1.67
	RV: GAGCTTCAGGGCTTCAGC	Pe	10.8	4.3					
	FWTAQ: CCGGGAAGCCCACCAT				1	6.4	2.8	10.3	3.8
	RVTAQ: TGAGCTCGACGCCCAGTT								
	TAQMAN: FAM-CACGTTCGTCAGAGTTGTAGCCAA								
TC9408	FW: TGGAAGTTTGCCGTGTACCTG	Am	15.1	14.0	1	1.35	ND**	3.04	3.85
	RV: AGGCCTTTCCAACACCAAAAAGTC	Iv	18.4	8.6					
		Co	19.1	16.5					
		Pe	20.0	11.8					
	FWTAQ: AAGGGCCACATCCAGAGA				1	9.2	5.2	21.0	7.0
	RVTAQ: TCAGCTGCCTTTTCTTTCAGTTT								
	TAQMAN: FAM-TCGCAGCGGAAGCCCAGCA								
TC9771	FW: TGTTTGTTTGCCTTCTTTCAACTG	Am	13.0	9.7	1	1.05	1.59	2.15	1.42
	RV: CGCCACCACTTGTAGAACTG	Iv	19.4	10.2					
		Co	12.2	5.9					
		Pe	13.6	6.7					
	FWTAQ: ATGCCGGCGTCGATAGAGT				1	13	3.6	38	9.8
	RVTAQ: CTAGGTGCCCTCCAATCGAA								
	TAQMAN: FAM-TGGATTCCCAGGAGCGACCC								
TC12018	FW: ACGCTTGTTATTGGGACGAC	Am	11.9	5.2	1	1.05	1.12	1.95	1.10
	RV: AGCACCGGTTGTTAAAGTGG	Pe	13.3	4.1					
BEACN33TR	FW: CAAGGACAAGGAATCGGAAAC	Am	10.1	8.3	1	0.75	0.55	2.85	0.60
	RV: ATGCCACAGGACAGAAGTGAC	Co	8.1	4.7					
		Pe	9.6	6.1					
18S	FWTAQ: CCTGAGAAACGGCTACCACATC								
	RVTAQ: GTGCCGGGAGTGGGTAATT								
	TAQMAN: VIC-AGGAAGGCAGCAGGCGCGC								

*BmiGI ID represents the identification number from BmiGI Version 2 except 18S which is the *R. microplus* 18S ribosomal RNA gene (GenBank Accession No. AF018656); †Primer 5′–3′ sequence of forward (FW) and reverse (RV) primers in the PCRs using the QuantumRNA 18S Internal Standards Kit and Competimers or the forward (FWTAQ) and reverse (RVTAQ) primers and Taqman probe (TAQMAN) from the real time PCRs; ‡Data from microarray experiments including acaricide (Am = amitraz, Iv = ivermectin, Pe = permethrin, and Co = coumaphos) which resulted in differential expression, the d statistic from SAM and fold-change ratio (FC);§Raw values normalized by division of the gene specific amplification product fluorescence by the 18S band amplification product fluorescence and expressed relative to untreated sample; ¶Untreated larvae; **Not Determined.

However, TC9004 was identified as a glutathione S-transferase (E-value = 2e−78) and the fold-change ratio (FC) showed 4.4-fold up-regulation upon coumaphos exposure in the microarray experiment and the SAGE data showed 5-fold up-regulation in the Munoz strain and up-regulation in the San Roman (Table 4). TC9004 is interesting, as glutathione S-transferase likely plays a role in a pyrethroid resistance sequestration mechanism in *Tenebrio molitor* (Kostaropoulos *et al*., 2001), organophosphate metabolism-based resistance in *Musca domestica* (Wei *et al*., 2001), and DDT metabolism-based resistance in *Anopheles gambiae* (Ranson *et al*., 2001). Table 4 shows that the SAGE experimental databases might not be as comprehensive as the microarray, possibly because not enough SAGE tags were accumulated to detect low abundance transcripts. Out of 24 SAGE library tag tally data points shown in Table 4, only two tag counts are > 2. In the transcript data shown in Table 4, the microarray data appears to be more sensitive in detecting differential expression than the SAGE experimental data. This could be corrected by deeper sequencing of the SAGE libraries, although at a significant cost tradeoff.

Relative quantitative RT-PCR. Nine of the differentially expressed transcripts were selected for analysis by relative quantitative RT-PCR and PCR primers designed from the corresponding sequences in BmiGI Version 2. Table 5 lists the forward and reverse PCR primer sequences and the acaricide treatment data from the exposures which resulted in statistically significant change in gene expression. The

densitometry results are normalized by division of the gene specific band densitometry value by the 18S band amplification product densitometry value. TC6631 had similar results in both the microarray and RT-PCR analysis. Additionally, TC6221 was generally consistent in that the microarray experiment showed only the amitraz treatment induced a significant difference in gene expression while the RT-PCR showed the highest expression in the amitraz-treated sample. However, the remainder of the microarray and RT-PCR comparisons produced mixed results. In TC7207, TC8948, TC9004, TC9408, TC9771, TC12018, and BEACN33TR the microarray and RT-PCR had at least one acaricide treatment set that was consistent. However, each of those BmiGI member transcripts also had at least one acaricide treatment where the RT-PCR did not detect differential expression while the microarray showed clear differences from the untreated sample. We had used relative quantitative RT-PCR with the QuantumRNA 18S Internal Standards Kit (See Experimental Procedures) to verify SAGE library tag tallies in a previous study (Guerrero *et al.*, 2007) and found the technique quite useful. However, those SAGE tag verifications were done with genes of relatively high expression levels as evidenced by the raw tag counts and the normalized densitometry values. In general, the transcripts examined by RT-PCR (Table 5) were likely low abundance and the PCRs proved more difficult to achieve consistency between runs. Both TC9004 and TC9771 had low SAGE tag counts which also indicated these were transcripts of relatively low abundance (Table 4). We selected three of the BmiGI entries from Table 5 for analysis by real time RT-PCR, selecting TC9004 and TC9771 because these had SAGE data and BlastX annotation (Table 4) and selecting TC9408 because this transcript showed the highest up-regulation in the coumaphos, permethrin and amitraz treatments and the second highest in the ivermectin treatment (Table 2). These results were also somewhat inconsistent for TC9004 in that the real time PCR found induction following each of the four acaricide treatments while the microarray only showed induction following the coumaphos and permethrin treatments (Table 5). The correspondence between real time PCR and microarray was reasonable for TC9408 and TC9771. Real time PCR analysis showed the amitraz treatment induced the highest gene expression differences compared to the untreated samples. Perhaps this should have been expected, as the strain possesses considerable amitraz resistance (Table 1). Even with these difficulties, TC9004 was very consistent in showing significant up-regulation upon exposure to coumaphos in the microarray and RT-PCR data of Table 5 and the Munoz and San Roman SAGE tag data from Table 4. Together with the annotation of TC9004 showing significant sequence similarity to glutathione S-transferase, the TC9004 gene product likely plays a role in responding to coumaphos treatment and supporting metabolic resistance to OP in this tick. Clarification of this role or its significance to survival of coumaphos treatment awaits expression and characterization of the TC9004 gene product.

Experimental procedures

Tick strains and treatment

The f23 generation of the San Alfonso strain of *R. microplus* was used. This strain originated from a ranch in Tabasco, Mexico in 2001, has been shown to be resistant to the formamidine amitraz, the organophosphate coumaphos, and the pyrethroid permethrin, and has been reared in the Cattle Fever Tick Research Laboratory (CFTRL) since November, 2002 following protocols described in Davey *et al.* (2008). The procedure was described in detail by the FAO (1971). Briefly, technical-grade acaricide was added to 2 parts trichloroethylene (Sigma, St. Louis, MO, USA) and 1 part olive oil (Sigma, St. Louis, MO, USA) to achieve the desired test concentration (Table 1). One ml of each dilution was applied evenly to a 7.5 × 9 cm piece of filter paper (Whatman #1, Whatman Ltd., Maidstone, UK). The trichloroethylene was allowed to evaporate from the filter paper for 2 h under a fume hood. The treated papers were then folded in half and sealed on the sides with clips. This formed a packet into which ca. 100, 14 d old larvae were placed after which the top of the packet was sealed with another clip. The packets containing larvae were held for 24 h in an environmental chamber at 27 ± 2 °C, 92% R. H. and a photoperiod of 12:12L:D. After this time, the packets were removed from the environmental chamber and all larvae suctioned into 1 ml freezer storage vials and frozen at –80 °C. A total of 1 g of larvae were used for each acaricide selection.

RNA extractions

Total RNA was isolated from 0.1 g larval material from each treatment group using two consecutive 40 s runs in the FastPrep-24 Tissue and Cell Homogenizer and Lysing Matrix D (Qbiogene, Irvine, CA, USA). A setting of 6.0 with a 3 min ice incubation between runs was used, followed by RNA isolation with the Fast RNA Pro Green Kit (Qbiogene) and subsequent lithium chloride precipitation using the Totally RNA Kit reagents (Ambion Inc., Austin, TX, USA). Approximately 50 μg total RNA in two 100 μl aliquots was treated with 2 μl of Turbo DNAse per Turbo DNA-free kit (Ambion Inc.) protocols. RNA integrity was verified by formaldehyde gel electrophoresis and staining in GelStar Nucleic Acid Gel Stain (Cambrex Bio Science, Rockland, ME, USA). Twenty μg of each DNA-free RNA from each treatment group was sent to NimbleGen Systems Inc. (Madison, WI, USA) for use in microarray hybridization.

Microarray design

Custom high-density single channel oligonucleotide arrays were constructed by NimbleGen Systems Inc. using 13 601 of the 13 642 members of BmiGI Version 2 and 14 perfect match 50-mer probes per BmiGI target. No mismatch probes were included on the arrays, although probes with randomly generated sequences were included. These random sequence probes were designed to match the melting temperature (Tm) of the other probes on the array and reflect the distribution of non-specific signal intensities for binding events to probes with approximately the same composition as the perfect match probes but with random sequences. Each microarray chip includes two in-slide replicates (spot replicates),

which were treated as technical replicates. Biological replicates assume that independently derived samples are used to assess variability between individual samples. Replication of biological samples is essential in order to draw significant conclusions that are valid beyond the scope of the particular samples that were assayed (Churchill, 2002). Technical replicates on the other hand, increase precision and provide a basis for testing differences within treatment groups. Because of the nature of limited RNA sources and the logistics of the collection and handling of *R. microplus* larvae, the ideal independent biological replicates were not available. Instead, we utilized pooled samples each consisting of several thousand tick larvae which provided the RNA source. We processed four technical replicates, i.e. repeated measurements of the same pooled *R. microplus* mRNA, consisting of two chip replicates with two spot replicates. The chip replicates were hybridized on different dates to different chips, while spot replicates indicate the same probe was spotted on different locations on the chip.

Array hybridization, scanning and image analysis/signal quantification

Five *R. microplus* samples were hybridized to separate microarrays at NimbleGen Systems Inc. The samples consisted of larvae from untreated San Alfonso (control) and four different San Alfonso larval samples exposed to coumaphos, permethrin, ivermectin, and amitraz at doses indicated in Table 1. The hybridization was done on separate days, resulting in a total of ten array hybridizations. The hybridized arrays were scanned with an Axon scanner (Axon Instruments, Union City, CA, USA) and image analysis done using GenePix software (MDS Analytical Technologies, Downingtown, PA, USA). NimbleScan software was used to place the design specific grid on the NimbleGen array images, quantitate the spots and extract the intensity raw values. The intensity raw values were normalized using quantile normalization to adjust intensity variations which arise from variation in the technology rather than from biological differences between the RNA samples. Quantile normalization is a complete data method and the goal of this type of normalization is to equalize the distribution of probe intensities for each array in a set of arrays (Bolstad, 2001). The gene calls were generated using the Robust Multichip Average (RMA) algorithm (Bolstad *et al.*, 2003; Irizarray *et al.*, 2003a,b). The software package tools used for normalization and gene calling are available through the Bioconductor project at: www.bioconductor.org. The normalized raw intensity data was log base 2 ($\log_2$) transformed to expand the scale of genes with low intensities while compressing the scale of genes with higher intensities. Since gene expression intensity values tend to be skewed towards an asymmetric distribution, the logarithmic transformation helps achieve a normal distribution of the data, which is a required assumption of most statistical analyzes. The fold change calculation using raw intensity value ranges from 0 to 1 for down-regulated genes and from 1 to infinity for up-regulated genes. The log transformation of the ratio data (fold change calculation) provides symmetry by treating the up and down regulated gene expression levels equally. The new ranges become-infinity to 0 and 0 to infinity for down and up regulation, respectively (Konradi, 2005). The microarray datasets have been submitted to the GEO database (www.ncbi.nlm.nih.gov/geo/; GEO accession number GSE10171).

Significance Analysis of Microarrays (SAM) Analysis

Once the normalized data files were reformatted, log transformed and loaded into Microarray Experiment Viewer (MeV Version 4.0, Dana-Farber Cancer Institute, Boston, MA, USA), SAM (Tusher *et al.*, 2001; Storey, 2002) was performed on the log base 2 normalized dataset. SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR) (Storey, 2002). SAM was used to select significant genes based on differential expression between control and acaricide treatment samples. The cutoff for significance is determined by a tuning parameter delta and one can also choose a fold change parameter to ensure that called genes change at least a pre-specified amount (Chu *et al.*, 2007). Threshold values of delta = 6.01 and a fold change ≥ 4.0 were used to separate significant from non-significantly differentially expressed ESTs. The design used under SAM is two-class unpaired design, where samples fall in either the control or acaricide treatment group. This was done four times, once for each acaricide. Due to the stringency of the selected delta and fold change parameters, SAM estimated the proportion of false positives as < 0.00001.

Microarray result verification

Hierarchical clusterings were performed using the MeV Version 4.0 algorithms. Several array results were selected for verification based on their level of differential expression and the amount of annotation available for their corresponding BmiGI sequence. DNA-free total RNA was used with random primers and the RETROscript Reverse Transcription Kit (Ambion Inc.) to generate cDNA. Relative quantitative reverse transcriptase-PCR was performed on nine differentially expressed transcripts with gene specific primers (Table 5), cDNA from each experimental sample, and the QuantumRNA 18S Internal Standards Kit (Ambion Inc.) using protocols described in Guerrero *et al.* (2007). Briefly, preliminary PCRs were performed to determine optimal annealing temperature for each gene specific primer pair, followed by a trial PCR to determine the number of cycles in the thermocycling program during which linear amplification was occurring. The QuantumRNA 18S Internal Standards Kit contains 18S primers and 18S Competimer primers modified so as to block extension by DNA polymerase. The 18S Competimer primers in this kit were used to modulate the amplification efficiency of the 18S cDNA, permitting its use for relative quantification during linear amplification of the PCR. The ratio of 18S and 18S Competimer primers in the presence of each gene specific primer pair was chosen such that both the gene specific primer target and the 18S were amplified to approximately the same levels.

For the real time PCR protocols, forward and reverse primers and TAMRA probes (Table 5) were designed using Primer Express Software v2.0 (Applied Biosystems Inc., Foster City, CA, USA) for each EST selected and the *R. microplus* 18S rRNA gene (GenBank Accession No. AF018656), which was to be used as a normalization control. Reactions for each EST and 18S rRNA gene were run in 96 well optical reaction plates (Applied Biosystems) using 25 µl total reaction volumes including primers, 250 nM TAMRA probe, TaqMan Universal PCR Master Mix without AmpErase UNG (Applied Biosystems) and cDNA for the untreated or acaricide-treated larvae. The EST-specific primer concentrations were 900 nM each while the 18S primers were 900 nM (forward primer) and 50 nM (reverse primer). The ABI Prism 7000 Sequence Detection System used a cycling profile of 10 min at 95 °C for AmpliTaq Gold enzyme activation and 40 cycles of 15 s at 95 °C

for denaturation and 1 min at 60 °C for annealing/extension. Analysis of fluorescence emission data from each cycle was done using ABI Prism 7000 SDS Software v1.1.

We also compared selected results from the coumaphos-exposed larvae microarray experiment to results from libraries of SAGE tags (Table 4) from a previous study of coumaphos-exposed larvae from the acaricide susceptible Munoz and the OP resistant San Roman strains of *R. microplus* (Guerrero *et al*., 2007). The Munoz strain is susceptible to all the acaricides used in this study while San Roman possesses 8-fold resistance to coumaphos, very high resistance to permethrin, and 3-fold resistance to amitraz (data not shown). SAGE tags were mapped onto members of BmiGI by in-house written Perl scripts (see Supporting Information). These scripts also searched using reverse complemented BmiGI sequences in the event the assumed 5′–3′ directionality was incorrect for some members of BmiGI. In BlastX analysis, a cutoff E-value of 0.001 was utilized to determine potential significant similarities between sequences.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Blast results for acaricide-induced differentially expressed members of BmiGI Version 2

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